AN APPRAISAL OF THE FUNCTIONAL SIGNIFICANCE OF THE INHIBITORY EFFECT OF LONG CHAIN ACYL-CoAs ON MITOCHONDRIAL TRANSPORTS

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1. Introduction

During the past few years, much attention has been paid to the inhibition by palmityl-CoA of some cellular enzymes and of mitochondrial transport systems. The particular interest of this inhibition is the possible role of acyl-CoA-sensitive enzymes in the mechanisms regulating lipogenesis and glucogenesis (for review see refs. [1–3]. The inhibition by palmityl-CoA of the citrate carrier in mitochondria [4] has also been discussed in terms of a possible regulation of fatty acid synthesis. It is conceivable that the rate of citrate exit from mitochondria may control the rate of acetyl-CoA formation through the activity of the citrate-cleavage enzyme located in the cytosol, but it must be noted that other transport systems, such as the ADP carrier [3, 5, 6] and the dicarboxylate carrier [4] are also sensitive to palmityl-CoA (or oleyl-CoA). The phosphate transport appears to escape this inhibition [6]. Although a number of papers have been published on this subject, the majority lack the quantitation necessary for an adequate evaluation of the physiological significance of the inhibitory properties of long chain acyl-CoAs.

In this paper, we compare the values of the inhibitor constant (K_i) for long chain acyl-CoAs of mitochondrial carriers concerned with the transport of ADP, phosphate, malate and citrate. We also examine the effects of the fatty acid chain length, and of the degree of unsaturation of the fatty acid moiety, on the inhibitory potency of the acyl-CoAs. The specificity of palmityl-CoA as an inhibitor of the ADP translocation is compared to that of atractyloside and carboxyatractyloside. Finally, we present data on the binding of [^{14}C]palmityl-CoA and [^{14}C]oleyl-CoA to mitochondria. The results taken together impose some reservation to the idea that the sensitivity of the mitochondrial transport systems to long-chain acyl-CoAs might be of physiological significance.

2. Methods

Rat liver mitochondria were isolated in 0.27 M sucrose buffered with 2 mM Tris, pH 7.4. Rats were killed after an overnight fast. Before use [^{14}C]malate (Amersham, Bucks, UK) and [^{14}C]citrate (Saclay, France) were purified by chromatography according to Myers and Huang [7]. [^{14}C]ADP was purchased from Schwarz (USA) and [^{32}P]phosphate from Saclay (France). Acyl-CoAs were synthesized from the corresponding chloride derivatives [8]. When the chloride derivatives were not commercially available, they were made from oxalylchloride, or thionyle chloride and the corresponding fatty acids. *Dicarboxylate transport* was tested as uptake of [^{14}C]malate [9] at 0°C. The medium was made of 0.1 M KCl, 25 mM Tris–MES (pH 6.5) 1 mM EGTA, oligomycin (2.5 μg/ml), antimycin (2.5 μg/ml) and 10^{-5} M rotenone. The reaction was initiated with [^{14}C]malate and stopped after 10 sec with 5 mM mersalyl, followed immediately

Abbreviations: CARBATR, Carboxyatractyloside (Gummiferin); MES, Morpholinoethane sulfonic acid; HEPES, Hydroxyethyl piperazine-ethane sulfonic acid; FCCP, Carbonyl cyanide P-trifluoromethoxyphenylhydrazine.

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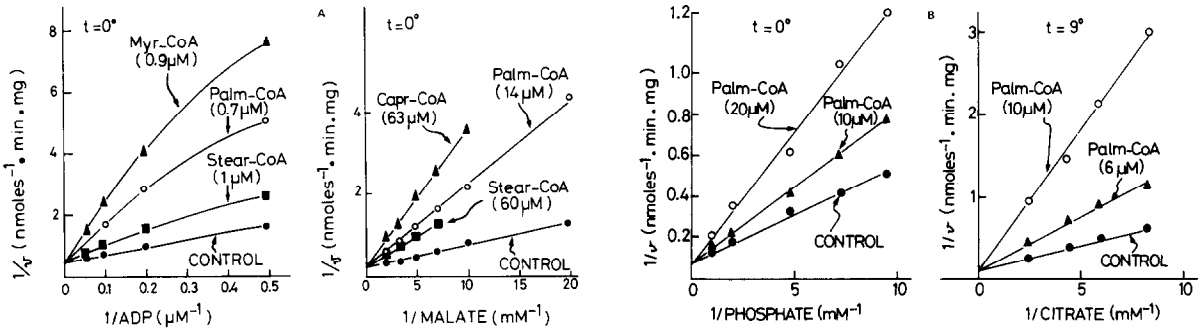

Fig. 1. Competitive inhibition by palmityl-CoA and other lang chain fatty acyl-CoAs of the mitochondrial transport of ADP, malate, citrate and phosphate.

by centrifugation in an Eppendorf 3200 microcentrifuge. The pellet was dissolved in 0.5 ml of a 4% sodium cholate (pH 10) and its radioactivity estimated by liquid scintillation counting. Correction was made for the sucrose permeable space by subtracting values obtained when mersalyl was added before malate. *Tricarboxylate transport* was measured as uptake of [^{14}C]citrate at 9°C with mitochondria preloaded with malate [10] in a medium similar to that used in experiments on malate uptake except that the pH was 6.8. The temperature of 9°C was chosen because of the low rate of citrate transport at 0°C. The exchange was initiated with addition of citrate and stopped with 20 mM benzene tricarboxylate after 10 sec. Determination of radioactivity in pellet and correction for sucrose-permeable space were made as in malate uptake experiments. *Phosphate transport* through the phosphate–hydroxyl carrier was tested as uptake of [^{32}P]phosphate at 0°C. The medium was made of 0.1 M KCl, 20 mM Tris–MES, pH 7.4, 1 mM EGTA, oligomycin (2.5 μg/ml) antimycin (2.5 μg/ml) and 10^{-5} M rotenone, 8 mM butylmalonate and 1 mM arsenite. The reaction was initiated by addition of [^{32}P]phosphate and stopped after 30 sec with 0.5 mM mersalyl followed by centrifugation. Extraction of pellet and correction for sucrose-permeable space was made as noted above. *Adenine nucleotide exchange* was measured at 0°C in 0.12 M KCl, 20 mM Tris, pH 7.4, 0.1 mM EDTA. The reaction was initiated by addition of [^{14}C]ADP and stopped after 30 sec with 6 μM carboxyatractyloside followed by centrifugation [11]. Correction for sucrose-permeable space was made by subtracting values obtained when carboxyatractyloside was added before ADP. For the transport of malate, citrate and phosphate, the amount of mitochondria ranged between 2.0 and 2.5 mg and the final volume was 1 ml. For the transport of ADP, the amount of mitochondria was 4–5 mg and the final volume 5 ml.

3. Results

3.1. *Sensitivity of the mitochondrial translocation of ADP, malate, citrate and phosphate to long chain acyl-CoAs*

As shown by the Lineweaver–Burk plots of fig. 1A and 1B, palmityl-CoA and other long chain acyl-CoAs inhibit competitively the uptake of [^{14}C]ADP, [^{14}C]malate, [^{14}C]citrate and [^{32}P]phosphate. Under the assay conditions used (see Methods), external [^{14}C]malate exchanges preferentially for internal phosphate [12] and (personal data); external [^{14}C]citrate exchanges for internal malate; and the uptake of phosphate proceeds through the phosphate/hydroxyl carrier [13]. The departure of plots from linearity in

Table 1
K_i values of mitochondrial transport systems for palmityl-CoA.

Transport system	K_i (Palmityl-CoA)
Adenine-nucleotide	1.0 to 2.5 × 10^{-7} M (4)*
Malate	7.1 to 9.5 × 10^{-6} M (5)
Citrate	3.2 to 4.5 × 10^{-6} M (2)
Phosphate	2.5 × 10^{-5} M (1)

* Number of experiments between parentheses.
Test conditions as described in Methods.

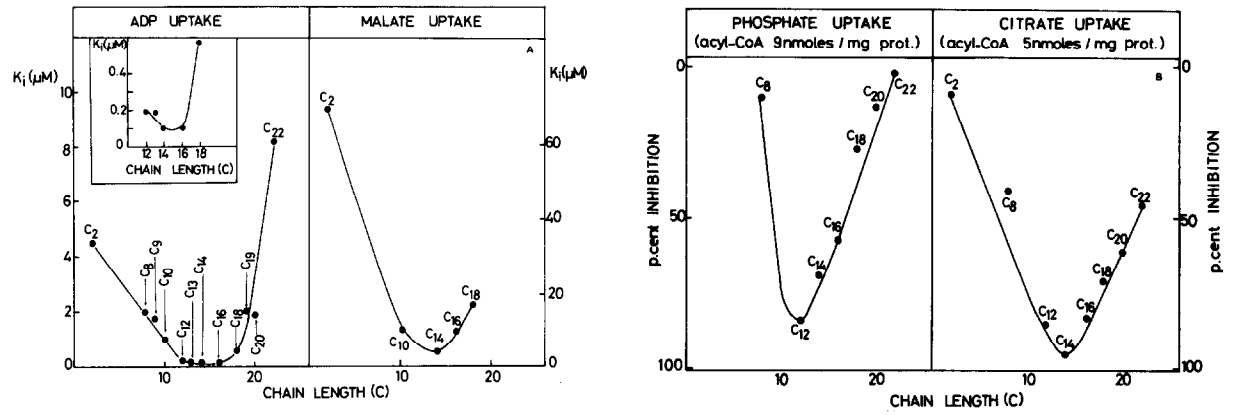

Fig. 2. Relation between the chain length of acyl-CoAs and inhibitory potency. Conditions as described in Methods. The K_i values were calculated from Lineweaver–Burk plots using for each acyl-CoA at least three different concentrations of the acyl-CoA.

the case of the ADP transport has also been found for the inhibition of the ADP transport by atractyloside [11] and appears to be due to the high affinity and the high capacity of binding of mitochondria for the inhibitor [14].

The K_i values of the above transport systems for palmityl-CoA differ markedly (table 1). The least sensitive carrier is the phosphate/hydroxyl carrier (K_i = 25 μM) which is 3–6 times less sensitive to palmityl-CoA than the tri- and dicarboxylate carriers (K_i = 4 μM and 8 μM respectively). The latter carriers are, in turn, more than 20 times less sensitive than the adenine-nucleotide carrier (K_i = 0.1 μM to 0.25 μM).

Since the binding capacity of mitochondrial membrane proteins for palmityl-CoA plays a critical role in the inhibition of the transport systems, the above values of K_i have been recalculated as nanomoles of palmityl-CoA per mg protein; this yields the following values: 0.1–0.2 nmole/mg protein for the ADP carrier; 2–4 nmoles/mg protein for the di- and tricarboxylate carriers and 12 nmoles/mg protein for the phosphate carrier.

Table 2
K_i values of the adenine-nucleotide transport system for some unsaturated fatty acyl-CoAs.

Chain length	Number of double bonds	Configuration	K_i (μM)
C_{18}: Stearyl-CoA	0	–	0.6
Oleyl-CoA	1	cis	0.1
Elaïdyl-CoA	1	trans	0.4
Linoleyl-CoA	2	cis–cis	0.3
C_{22}: Behenyl-CoA	0	–	8.2
Erucyl-CoA	1	cis	0.4

Conditions as described in Methods.

3.2. *Effect of the fatty acyl chain length and of the degree of unsaturation on the inhibitory potency of fatty acyl CoAs*

Data in fig. 2A shows how the K_i of the ADP carrier and the malate carrier for acyl-CoA depends on the length of the fatty acid moiety whatever the parity of the number of carbon atoms may be. Data in fig. 2B relates the percentage of inhibition by acyl-CoAs for phosphate carrier and citrate carrier to the acyl chain length. In all cases the maximal inhibitory effect was found for chain lengths between C_{12} and C_{18}. For a given chain length, the degree of unsaturation and the stereoisomery of the double bonds did not appear to play a critical role. Typical data for different fatty acid derivatives with a chain length of 18 carbon atoms are shown in table 2. However, there may be exceptions to this general rule as shown for two acyl-CoAs with 22 carbon atoms; the unsaturated one, namely erucyl-CoA (13-cis, docosenoyl-CoA) is twenty times more effective than the saturated one i.e. behenyl-CoA. One may wonder whether the toxic properties of erucic acid [15] are linked to the high inhibitory potency of erucyl-CoA. Since the solubility of long chain acyl-CoAs increases when temperature is raised, the inhibitory effect of palmityl-CoA on the ADP translocation has been measured comparatively

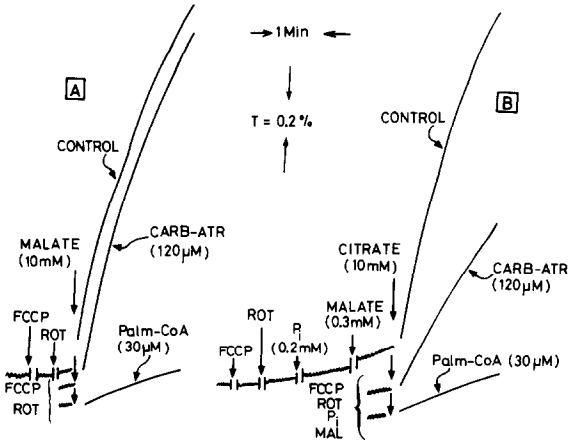

Fig. 3. Effect of palmityl-CoA and carboxyatractyloside on the reduction of endogenous NAD(P) by malate and by citrate. Rat liver mitochondria (4 mg) were suspended in 3.2 ml of 0.27 M sucrose, 1 mM EDTA, 10 mM HEPES, 5 mM Tris–HCl, final pH 7.2, temperature 25°C, 1 μM FCCP, 6 μM rotenone. Mitochondria were incubated for 3 min with FCCP to remove endogenous substrates and oxidize pyridine nucleotides and then rotenone was added to block the respiratory chain. The reduction of intramitochondrial NAD(P) was measured in a double beam spectrophotometer at 340–374 nm.
Trace A – Reduction of NAD(P) upon addition of malate.
Trace B – Reduction of NAD(P) upon addition of citrate in the presence of small amounts of phosphate and malate.

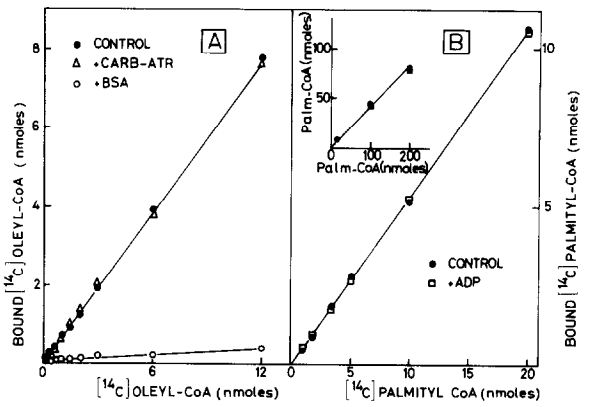

Fig. 4. Binding of [^{14}C]oleyl-CoA and [^{14}C]palmityl-CoA to rat liver mitochondria. A – Rat liver mitochondria (2.1 mg protein) were incubated for 30 min at 0°C in 4 ml of 0.12 M KCl, 0.3 mM EDTA, 5 mM HEPES, pH 6.8, 5 μM rotenone, 2 μg antimycin and 20 μg oligomycin. When present, carboxyatractyloside was 1 μM and bovine serum albumin 20 mg/ml; B – Same conditions except that 7.3 mg of mitochondrial protein were used and the final volume was 10 ml. ADP was used at a concentration of 20 μM (cf. inset). For A and B valused plots in abscissa and ordinate refer to the total amount of acyl-CoA added in the medium and bound to mitochondria.

at 0°C and at 9°C; no significant difference in inhibition was found.

3.3. *Cross reactivity between the adenine-nucleotide carrier and the citrate carrier*

It has been recently reported that the citrate carrier and the adenine nucleotide carrier in mitochondria are able to interact with common ligands. For example phosphoenolpyruvate which behaves as a substrate for the citrate carrier, is also transported by the ADP carrier, although its affinity for the ADP carrier is rather low [16]. Furthermore, atractyloside, a well-known competitive inhibitor of the ADP translocation, has been found to inhibit, at high concentrations, the transport of citrate [17]. As shown in this paper the ADP carrier and the citrate carrier are both inhibited by long chain acyl-CoAs. To examine the cross reactivity of both carriers we have examined the effect of the citrate uptake of carboxyatractyloside (Gummiferin), a powerful non-competitive inhibitor of the ADP translocation [11]. In the experiment presented in fig. 3B in which citrate uptake was assayed by the spectrophotometric method of Chappell and Haarhoff [18] the presence of 120 μM carboxyatractyloside inhibited nearly half of the rate of citrate uptake. The K_i for carboxyatractyloside of the citrate carrier as determined by direct uptake of [^{14}C]citrate was 17 μM.

It must be recalled that the K_i of the ADP carrier for carboxyatractyloside is less than 10 nM [11]. Nevertheless the inhibition by carboxyatractyloside of the citrate carrier is significant in view of the total lack of effect of carboxyatractyloside on the dicarboxylate carrier (fig. 3A). It must be pointed out that the binding affinity and capacity of mitochondria for carboxyatractyloside assayed with [^{35}S]carboxyatractyloside [11] is not decreased by concentration of palmityl-CoA as high as 20 μM (unpublished data).

3.4. *Binding of [^{14}C]palmityl-CoA to mitochondria*

The non-saturating character of the binding of [^{14}C]palmityl-CoA to mitochondria is illustrated in fig. 4. This may be contrasted with the saturation plateau observed for the binding of carboxyatractyloside. For example the saturation of rat liver mitochondria by carboxyatractyloside is attained at concentrations of carboxyatractyloside less than 1 μM using 5 mg of

mitochondrial protein [19]. In contrast, the ratio of bound acyl-CoA to free acyl-CoA remains practically constant and close to 2 for concentrations of oleyl-CoA ranging from 0.1 μM to 3 μM; it was of 1.2 to 1.4 for palmityl-CoA up to 20 μM. Interestingly, whereas bound [^{14}C]palmityl-CoA is removed by bovine serum albumin, presumably by competition through unspecific binding of its acyl moiety [20], it is not displaced by ADP nor by carboxyatractyloside.

4. Discussion

As shown in this paper, mitochondrial transport systems fall into three groups (ADP, tri- and dicarboxylate anions, and phosphate) depending on their susceptibility to the inhibitory effect of long chain acyl-CoAs. Since the sensitivities of these three groups to palmityl-CoA differ by a factor of 3 up to 40 (K_i of the ADP carrier 0.1–0.2 μM, K_i of the di- and tricarboxylate carriers 4–8 μM, K_i of the phosphate/OH^- carrier 25 μM), one wonders whether the inhibitory properties of palmityl-CoA may have physiological significance. For example, a graded accumulation of palmityl-CoA might result in a stepwise inhibition of these carriers, beginning by the ADP carrier, then reaching the tri- amd docarboxylate carriers and finally the phosphate carrier. However, such a situation appears unlikely since a partial inhibition of the tricarboxylate carrier is obtained at concentrations of palmityl-CoA that would totally inhibit the ADP carrier. Some other data also impose some reservation to the idea that the inhibition by long chain acyl-CoAs of the mitochondrial carriers might by of physiological significance: 1) the acceptance capacity of biological or artificial membranes for acyl-CoA is very high and unspecific; for example bilayers of dimyristyllecithin can incorporate as much as 20 palmityl-CoA molecules per 100 lipid molecules [21]; in this study we found a high partition coefficient of palmityl-CoA and oleyl-CoA between mitochondria and the medium (ranging between 1.2 and 2); 2) the inhibitory efficiency of long chain acyl-CoA does not depend on the parity of the number of carbon atoms in the acyl chain although most of the fatty acids found in the cell have an even number of carbon atoms; 3) although the inhibition caused by palmityl-CoA to the transport of a given anion is competitive, the binding of palmityl-CoA to mitochondria is hardly modified by the addition of this anion. This behavior may be contrasted with the stoichiometric displacement by ADP of bound atractyloside [22], a competitive inhibitor of the ADP transport; 4) Vaartjes et al. [2] have calculated that the concentration of long chain acyl-CoA in rat liver cell is of 0.8 nmole/mg protein. By direct measurement of the ADP translocation we have determined a K_i value for palmityl-CoA of 0.1–0.2 nmole/mg protein which is well below the above mentioned concentration of palmityl-CoA in the cell; it is therefore probable that there exists some cellular compartmentation which renders the ADP translocator not accessible to long chain acyl-CoAs in vivo.

An unexpected result reported in this paper is the inhibitory effect of carboxyatractyloside on the citrate carrier. Although relatively weak, the carboxyatractyloside inhibition of the citrate transport is not insignificant, and it may be contrasted to the complete lack of effect of carboxyatractyloside on malate transport. A possible explanation may be the proximity of the ADP and citrate carriers on the inner mitochondrial membrane.

The pleiotropic effect of long chain acyl-CoAs on mitochondrial carriers is probably due to hydrophobic interactions between the acyl moiety of the acyl-CoAs and the carriers. The characteristic hydrophobicity of each carrier would thus determine, at least in part, the efficiency of its interaction with acyl-CoA. The ineffectiveness of the water-soluble short chain acyl-CoAs supports this hypothesis. On the other hand, the fact that acyl-CoAs with a chain length higher than C_{18} are less effective than those with 14 to 18 carbon atoms may be related to processes of intercalation and lateral diffusion between the paraffinic chains of membrane phospholipids [23, 24]. Such processes are facilitated when the added acyl-CoAs have the same chain length as the acyl moiety of membrane phospholipids, which is obviously the case as far as the inner mitochondrial membrane is concerned [25]. Wojtczak et al. [26] have shown previously that free long chain fatty acids are inhibitors of ADP translocation. The fact that the CoA derivatives are more potent inhibitors than free fatty acids could be a result of their different physical parameters relating to their micellar size, to the ease of the fusion of the micelles with the mitochondrial membrane and to differences in mobility within the membrane, rather than to a specific physiological interaction of the acyl-CoAs.

The non linear double reciprocal plots obtained for the kinetics of ADP transport at different concentrations of palmityl-CoA (fig. 1) deserves some comments. As discussed by Henderson [14] for enzyme kinetics and tightly bound inhibitors, the departure of plots from linearity is due to the large variation of the free inhibitor concentration when the total inhibitor concentration is fixed and that of substrate varies. However this explanation does not apply satisfactorily to the inhibition of the ADP transport by palmityl-CoA since bound [^{14}C]palmityl-CoA is not significantly displaced by ADP to give free [^{14}C]palmityl-CoA. A possible explanation is that the bound palmityl-CoA which is located in the close neighbourhood of the ADP carrier interacts with the carrier when the latter is unloaded; on the other hand, it would be displaced away from the carrier in the core of the membrane (without being released in the medium) when the ADP carrier becomes loaded upon addition of ADP.

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